Immunohistochemical localization of Vesicular Acetylcholine Transporters and Choline Acetyl Transferase Activity in Murine and Human Immune Tissues

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Abstract **- The cholinergic innervation of lymphoid organs, through autonomic nerves is not well understood. The aim was to localize the cholinergic nerve endings in immune tissues of Balb/C mice, Wistar rats and humans using antibodies of vesicular acetylcholine transporter proteins (VAChT) and choline acetyl transferase (ChAT) enzyme found in the nerve terminals of cholinergic system. Immune tissues were processed for Haematoxyline & Eosin staining and immunohistochemistry. Thin sections were labeled by primary antibodies, anti- VAChT, anti-ChAT and biotinylated anti-rabbit IgGs. Labeled StreptAvidin Biotin method was applied using Diaminobenzidene chromogen and by light microscopic visualization. Skeletal muscle was used as positive control. Photomicrographs were developed using a binocular light microscope equipped with a digital camera (Dino capture, Taiwan), which display the images in the monitor screen of a personal computer and images were analyzed depending on the intensity of immunostaining. Immunoreactive axon terminals of cholinergic neurons were counted. 0 (No staining), 1+ (focal staining, less than 3), 2+ (focal to diffuse staining, 4 to 6), 3+ (diffuse staining, 7 to 10). Immunoreactivity to VAChT antibody was high in capsule and red pulp while absent in white pulp of spleen. The capsular & septal regions of thymus showed 2+IR and the capsule & subcapsular areas of lymph nodes showed 1+IR to VAChT. The VAChT was not localized in liver and Peyer's patches. The IR of ChAT was similar to the distribution of VAChT in spleen, lymph nodes, thymus and absent in Peyers' patches. Also the IR of VAChT and ChAT in immune tissues of the three species were similar. The close proximity of the distribution of VAChT and ChAT in capsular and perivascular supporting framework of these tissues confirms that the immune tissues receive cholinergic innervation and is through capsular and perivascular supporting framework of these tissues.**

Keywords- Immunohistochemistry, Vesicular acetylcholine transporter, Choline acetyl transferase, immune tissues

I. INTRODUCTION

Autonomic innervations of different lymphoid organs and the bidirectional communication between nervous system and immune system is well documented [1,2,3]. Innervation by sympathetic noradrenergic fibres of lymphoid organs has been fairly well studied by many researchers. Yet the cholinergic nerves and the specific acetylcholine receptor subunit distribution in immune tissues are not well characterized. Parasympathetic cholinergic innervations have been localized in normal thymus of rat in a documented study [9] which showed the entry of AChE-positive nerve fibres in the capsular region towards the parenchyma, and running parallel to blood vessels to be distributed in the medulla. In cortex, AChE-positive nerve fibres have been localized in the subcapsular areas and the corticomedullary junctional areas [8,9]. In addition, embryonic studies have shown the distribution of AChE-positive nerve fibres in the capsule, interlobular septa and in subcapsular and cortico-medullary areas of rat thymus, around the 18th day of foetal life [18]. The density of these fibres increased during development and the specific AChE activity was detectable around the 19th day of gestation. This suggests that the nerves might be potentially involved in the regulation of the organ activity by acting on thymocytes during the embryonic period [18]. Another study done applying ChAT immunohistochemistry showed the localization of fine perivascular nerve fibres in thymic parenchyma [4]. Further, an electrophysiological investigation of thymic vagal innervation of rat showed that the fibres primarily consist of non-myelinated C-fibres and the lobes of the thymus are supplied bilaterally by fibres from cervical vagus [25].

The relationship between sympathetic and parasympathetic intra-parenchymal nerve distribution in thymus has been studied in a rat model using experimental denervation techniques. It was observed that sympathetic, parasympathetic and somatic nerves had a distribution of 60%, 30% and 10% of total nerve fibres, respectively [5].

The autonomic innervation of spleen has been traced via the trans-synaptic retrograde tracer Pseudorabies virus (PRV) and the results indicated anatomical connections of spleen by parasympathetic part of the autonomic nervous system [6]. Parasympathetic

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innervation of human foetal spleen showed that the nerve fibres found to be closer to sympathetic nerves & running in the proximity of hilar splenic vessels which irradiate into the parenchyma [18]. It has been also shown that the density of intraparenchymal nerves was greater in the foetal spleen compared with the adult.

The neuroanatomical evidence of parasympathetic input of lymph nodes is lacking up today. Therefore by demonstrating the in-situ localization of cholinergic nerve endings in immune tissues, the cholinergic innervation could be clearly understood.

The recent development of antibodies against the subtypes of acetylcholine receptors, vesicular acetylcholine transporter protein (VAChT) and choline acetyl transferase (ChAT) permits a greater reliability in identification of cholinergic innervations. VAChT has been localized in the synaptic vesicles of cholinergic axon terminals and ChAT being the most widely studied has revealed the prominent role of cholinergic transmission in Alzheimer's disease and other disorders of the nervous system [7]. The objective of this study is to investigate the distribution of cholinergic nerve endings in the immune tissues ie.thymus, liver, spleen, Peyer's patches and lymph nodes of Wistar rats, Balb/C mice & human by immunohistochemistry.

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II. METHODOLOGY

A. Ethical clearance

The ethical clearance was obtained by the Ethics Review Committee of Faculty of Medical Sciences, University of Sri Jayewardenepura, Sri Lanka for the animal tissue isolation and to collect fresh post-mortem human lymphoid tissues.

B. Lymphoid tissues from Balb/C mice and Wistar rats

Adult male Balb/C mice of 7-8weeks (n=5) and Wistar rats aged two months (n=5) were obtained from the Medical Research Institute, Colombo and housed in the Animal house of the Faculty of Medical Sciences, University of Sri Jayewardenepura under specific pathogen-free conditions in filter top cages. Isolation of lymphoid organs and tissues were performed after euthanizing the animals with chloroform**.**

The axillary, cervical, inguinal and mesenteric lymph nodes, thymus, spleen, ileal Peyer's patches were removed and transferred immediately to 10% formal saline fixative and left for 3-4 days for complete fixation.

C. Fresh Lymphoid tissues from humans

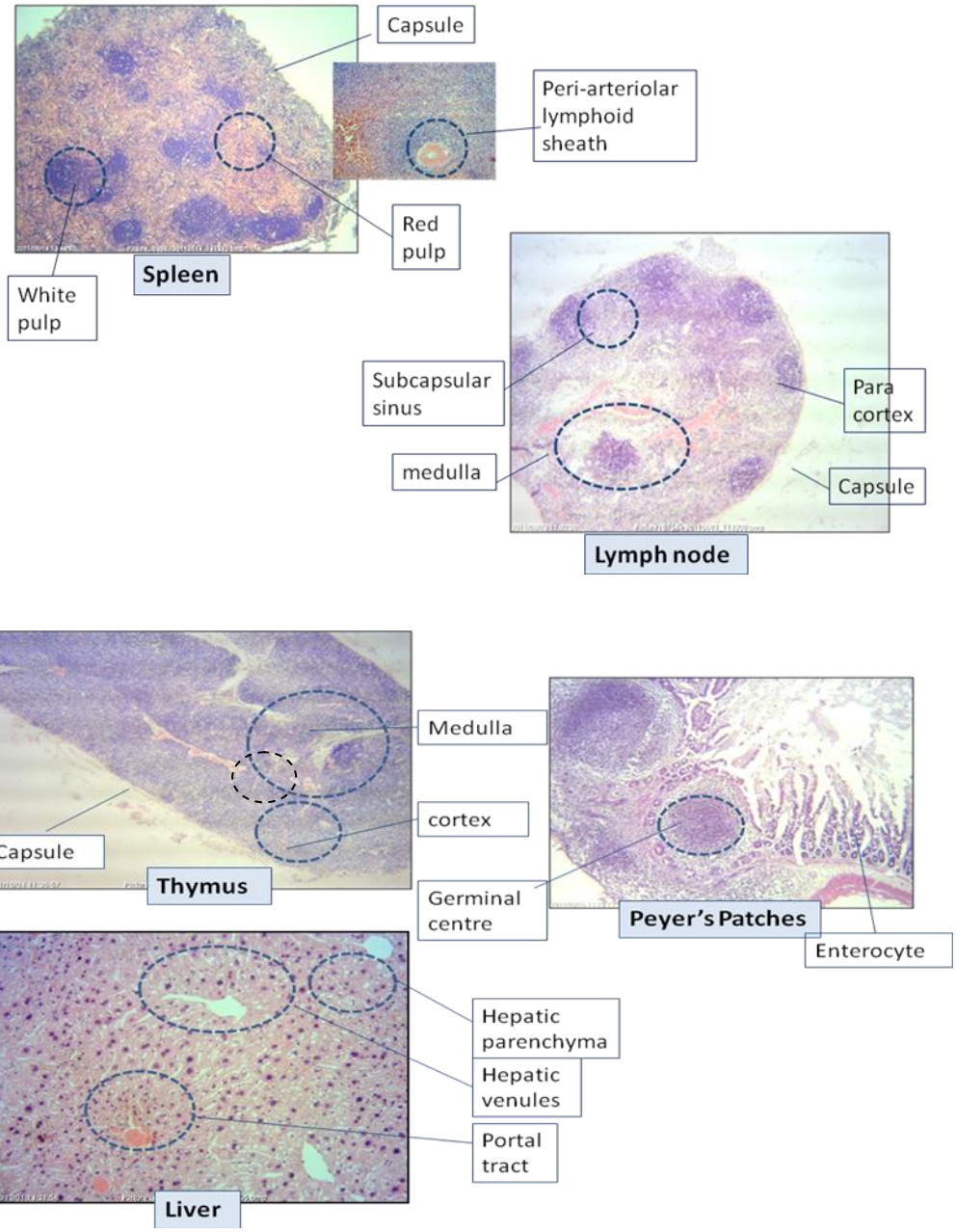
The fresh human lymphoid tissues were obtained at the time of the post-mortem from the Judicial Medical Officer of Colombo South Teaching Hospital. The autopsy samples were collected from five humans (M=4, F=1) aged between 20 to 60 years after obtaining informed written consent from the next of kin. Those who had died from infectious diseases and persons with autoimmune diseases were excluded. Samples collected were from the spleen, ileal Peyer's patches, and the cervical, para-aortic and mediastinal lymph nodes. The posterior part of the tongue towards the palate was also sampled.

All the collected tissues were immediately fixed in 10% formal saline in separate containers and transported carefully to the histology laboratory of the Department of Anatomy, Faculty of Medical Sciences, University of Sri Jayewardenepura. The tissue samples were washed in running tap water to remove blood stains and other artefacts and kept in formalin fixative for 3-4 days for complete fixation in closed containers.

D. Preparation of tissues for immunohistochemistry

After complete fixation, the immune tissues were imbedded in wax and then 3-4μm sections were cut and processed for routine histology techniques and stained with Haematoxylin & eosin. H & E stained slides were air dried and examined under the light microscope (Binocular, Olympus, UK, x40).

E. The sub-cellular compartments of different immune tissues



F.Immunohistochemical staining

The lymphoid tissue sections of 3-4μm thickness were cut from the paraffin blocks of the selected immune tissue and placed on poly-L-lysine coated microscope slides (Dako, Denmark) air dried and placed in an incubator at 56-60^{o}C overnight for fixation.

The slides fixed with lymphoid tissues were cleared in xylene and rehydrated in 2 changes of 95% ethyl alcohol. Subsequently**, t**he endogenous peroxidase blocking was done with methanol containing 3% Hydrogen Peroxide for one hour and placed in Phosphate Buffered Saline (PBS,0.1M,pH=7.4) wash bath for 15 minutes for further rehydration. Heat induced antigen retrieval was then performed in a microwave-resistant plastic staining jar containing antigen retrieval solution (0.1 M Citrate buffer, pH=6) for 5 min. on high power (~700 watts) in a microwave oven. The pre-cooled tissue sections were treated with 3% v/v H_2O_2 for further blocking of endogenous peroxidases and rinsed and washed twice in PBS bath for 10 minutes in each. The slides were pre-incubated with 5% Bovine serum albumin in 0.1M PBS for 10 min. prior to the addition of primary antibody to reduce non-specific binding of antibody.

The primary antibodies used were directed against the Vesicular Acetylcholine transporter Protein and Choline Acetyl transferase enzyme (Abcam, UK & Sigma Aldrich, USA) and were used at a dilution of 1:3000 in 1% BSA in 0.1M PBS. A positive control slide (a tissue known to contain the antigen under study) was also included in each experiment.
One hundred µl of the primary antibody was added to each tissue slides and the appropriate slides were incubated in a humidifying chamber in the refrigerator for 18-20hrs. After the stipulated incubation time period the slides were taken out and allowed to drain and reacted with biotinylated peroxidase labelled anti-rat IgG (dilution 1:750, Sigma Aldrich, USA) and incubated in a humidity chamber for 30 min. at room temperature.
Diluted Extravidin peroxidase (dilution 1: 200, Sigma Aldrich, USA) was applied to the tissue sections and incubated for 20 minutes in the humidity chamber at room temperature. Fresh solution of diamino benziedene (DAB) used as a chromogenic peroxidase substrate, was prepared by reconstitution of 20mg of DAB tablets in 5ml of distilled water. Enough drops of freshly prepared substrate (DAB solution, Sigma Aldrich, USA) mixture were applied to cover the tissue section. The slides were incubated for 5-10 min. until desired brown colour reaction was observed and monitored with the microscope and terminated by washing in the distilled water.

For counterstaining, Mayer's haematoxylin was applied and left for 2 minutes. Then the excess stain was washed off and the slide rack was dipped in to the ammonium hydroxide solution. The slides were dipped for several times until the desired bluish colour change observed in the tissues. The stained tissue sections underwent a series of ethanol and xylene washes for dehydration and clearing. Finally the immune tissues were cleared from xylene baths and mounted with DPX.

The prepared slides were observed under light microscope to identify the possible immunoreactivity (IR) found in different sub-cellular compartments of each immune tissues. The slides with less background staining were selected to produce photomicrographs.

G. Image collection and processing

The photomicrographs were developed using a binocular light microscope equipped with a digital camera (Dino capture, Taiwan), which display the images in the monitor screen of a personal computer. Images of lymphoid tissues were captured by the digital camera in different magnifications (x40, x100, and x400) and were magnified in the computer screen in x640 x480 magnification. Ten fields were chosen in x400 magnification and the immunostained sites of immune tissues were counted and average number of IR sites was calculated. Photomicrographs were taken only from the slides showing less background staining with clear ultra structural features.

IHC slides were prepared for each immune tissue of all three species. Five tissue sections were taken from each tissue and the immunoreactivity was analysed extensively in different magnifications (x40, x100, and x400) of light microscope and under the oil immersion (magnification- x1000).

Qualitative computerized image analysis was performed. The computerized images were analyzed depending on the intensity of immunostaining which was determined based upon a score of 0 (No staining), 1+ (focal staining, > 30% cells), 2+ (focal to diffuse staining, 30% > 60% cells), 3+ (diffuse staining, 60>100% of cells). Not less than 100 cells were evaluated in non-overlapping high power field of the light microscope (x400) as described in Schaulder et al, 2008.The scoring was adapted for the IR of cholinergic fibres. Immunostained axon terminals of cholinergic neurons were counted. 0 (No staining), 1+ (focal staining, > 3), 2+ (focal to diffuse staining, 3> 6), 3+ (diffuse staining, 6> 10).

III. RESULTS

A. Localization of the VAChTs in immune tissues of Balb/C mice, Wistar rats and humans

The positive control slides of skeletal muscle tissue of postmortem human showed strong dark brown spotted areas of sarcoplasmic membrane which were IR to VAChT.

1) Distribution of VAChT in Spleen

The VAChT antigens were localized considerably in the splenic capsule, trabeculae and venous sinusoids in the red pulp of the murine and human immune tissues.. The red pulp and capsule expressed a higher IR of VAChT and the rest of the tissue expressed a very low IR in those tissues. .

2) Distribution of VAChT in Lymph node

The expression of VAChT on the capsule and subscapsular sinus were relatively high (2+) compared to the cortex and medulla of the lymph nodes of the three different species.

3) Distribution of VAChT in thymus

In thymus the IR of VAChT on the capsule and septa was seen in the thymuses of mice & rats and they were located along the interlobar septa and very few within the cortex.

4) Distribution of VAChT in Peyer's Patches

In Peyer's patches the IR of VAChT was not found in the germinal centre, mantle zone and other related tissues. Almost similar results obtained in the three species.

B. Localization of the ChAT enzyme activity in immune tissues of Balb/C mice, Wistar rats
Cerebral cortex of rat brain was used as positive control to visualize the IR of ChAT enzyme. It was located with brown staining within the cytoplasm of CNS of neurons

1) Localization of ChAT in Spleen
The splenic capsule, red pulp and the trabeculae of venous sinusoids showed 1+ IR of ChAT enzyme in the tissues of Balb/C mice and Wistar rats.

2) Localization of ChAT in lymph node
The IR of ChAT on the capsule of lymph node, the subcapsular sinus was observed as 1+ while the cortex and medulla expressed very low or absence of IR of ChAT in both murine species.

3) Localization of ChAT in thymus
The IR of ChAT on the capsule and septa was high in thymus and the cortex and the medulla were not localized with ChAT.

4) Localization of ChAT in Peyer's patches
The IR of VAChT in the germinal centre, mantle zone and in the relative tissues was very low and it was shown to be absent in those regions.

Positive control slides of skeletal muscle tissue of VAChT and ChAT

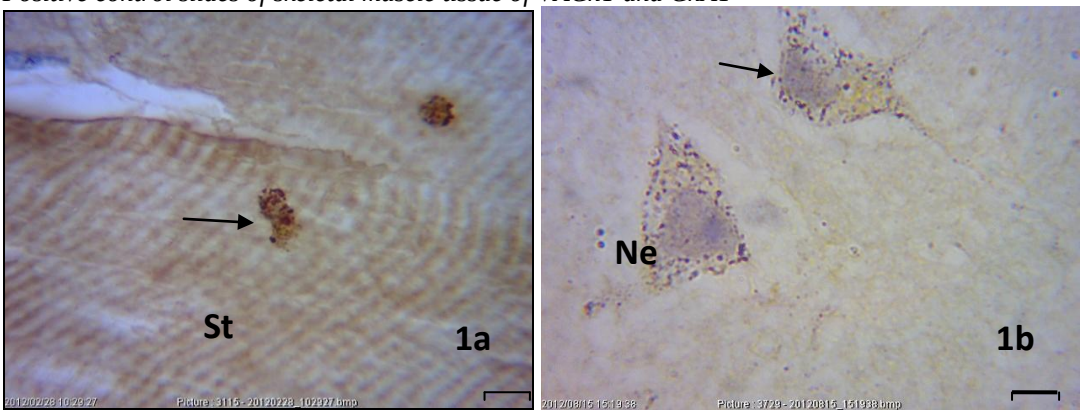


Plate 1a- Immunohistochemical identification of Vesicular Acetylcholine Transporters (VAChTs) in paraffin embedded sections of skeletal muscle tissue of human used as positive control. The image shows the strong dark brown spotted areas on the sarcoplasmic membrane which displays the positive staining of VAChT. These spots are indicated by the arrows Scale bar = 10 µm.
Plate 1b – Immunohistochemical identification of paraffin embedded sections of Cerebral cortex recovered from Wistar rats shown as the positive control. The image shows the dark brown spotted cytoplasm of the neurons which displays the positive staining of ChAT enzyme. Those stained neurons are indicated by the arrows Scale bar = 10 µm. Abbr. St- Striations, Ne- Neuron

Photomicrographs shows the immunoreactivity of VAChT and ChAT in different immune tissues

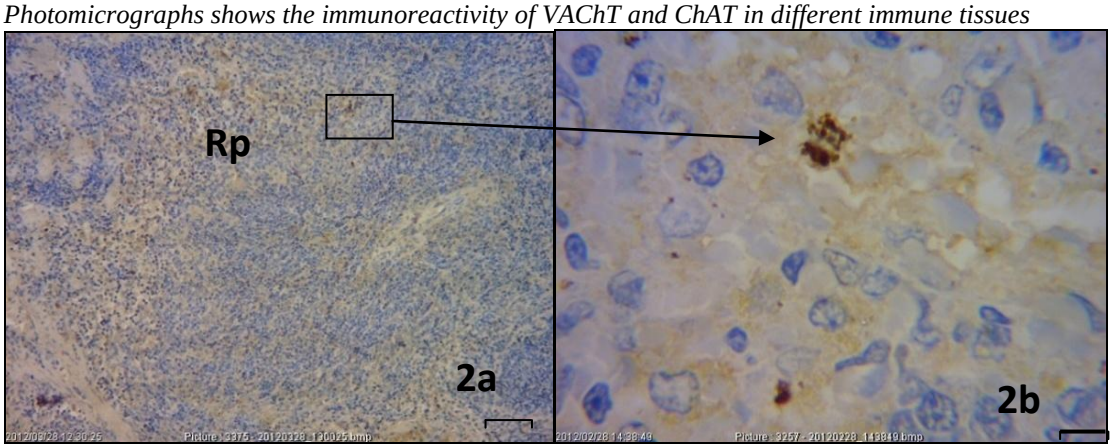


Plate 2 – Immunohistochemical identification of VAChTs in paraffin embedded sections of spleen of Balb/C mice. Fig 2a- shows the dark brown colour spots localized in the in the red pulp region indicated within the square has been magnified in Fig 2b- to identify clearly the immunoreactive site of VAChT. Scale bars = 90µm (Fig a) and 10µm (Fig b), Rp- Red pulp

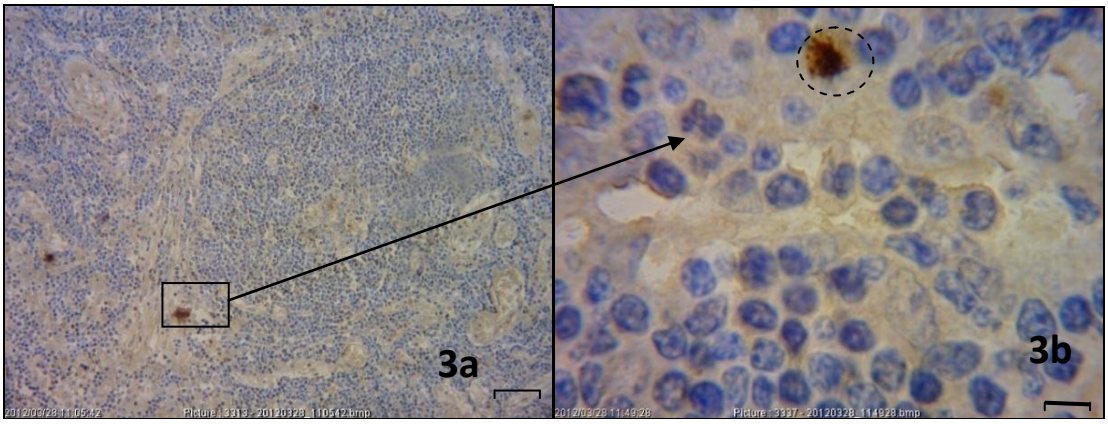


Plate 3 – Immunohistochemical identification of VAChTs in paraffin embedded sections of lymph node of Balb/C mice. Fig 3a- shows the dark brown colour spots localized in the in the subcapsular region indicated within the square has been magnified in fig 3b- indicated by arrows. Scale bars = 90µm (Fig 3a) and 10µm (Fig3b)

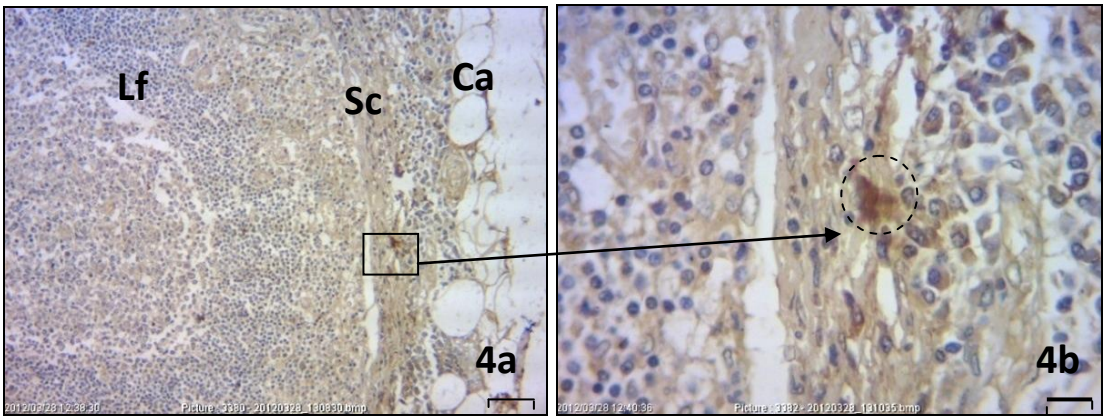


Plate 4 – Immunohistochemical identification of VAChTs in paraffin embedded sections of spleen of humans, Fig 4a- shows the dark brown colour spots localized in the in the red pulp region indicated within the square has been magnified in fig 4b. Scale bars = 90µm (fig a) and 10µm (fig 4b)

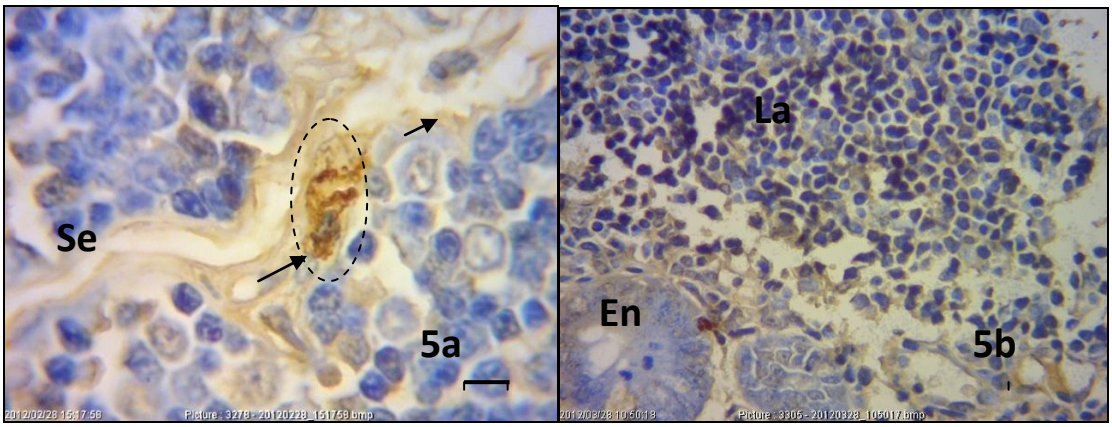


Plate 5a – Immunohistochemical identification of VAChTs in paraffin embedded sections of thymus of Wistar rats. Image shows the dark brown colour spot localized in the in the septa. Scale bar= 10µm , Abbr. Se- Septa

Plate 5b – Immunohistochemical identification of VAChTs in paraffin embedded sections of Peyer's patches of Balb/C mice. Image shows the absence of immunoreactive staining of VAChT. Scale bars = 90µm, Abbr.- La- Lymphoid aggregations, En- Enterocytes

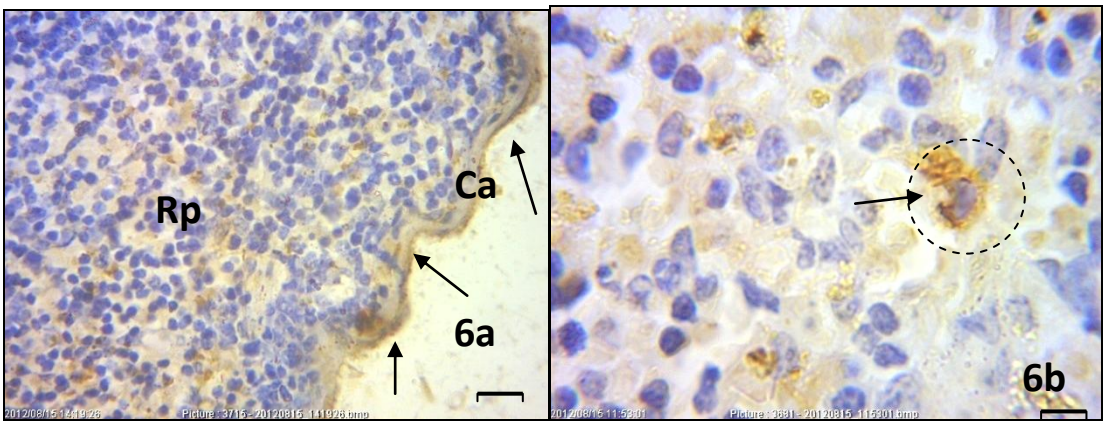


Plate 6 – Immunohistochemical identification of ChAT in paraffin embedded sections of spleen of Balb/C mice. Fig 6a- shows the dark brown colour stained sites localized in the in the red pulp and the capsular region indicated by arrows, the square indicated in Fig 6a has been magnified in Fig 6b to identify clearly the immunoreactive site of ChAT. Scale bars = 90µm (fig 6a) and 10µm (fig 6b) , Abbr. Rp- Red pulp, Ca- Capsule

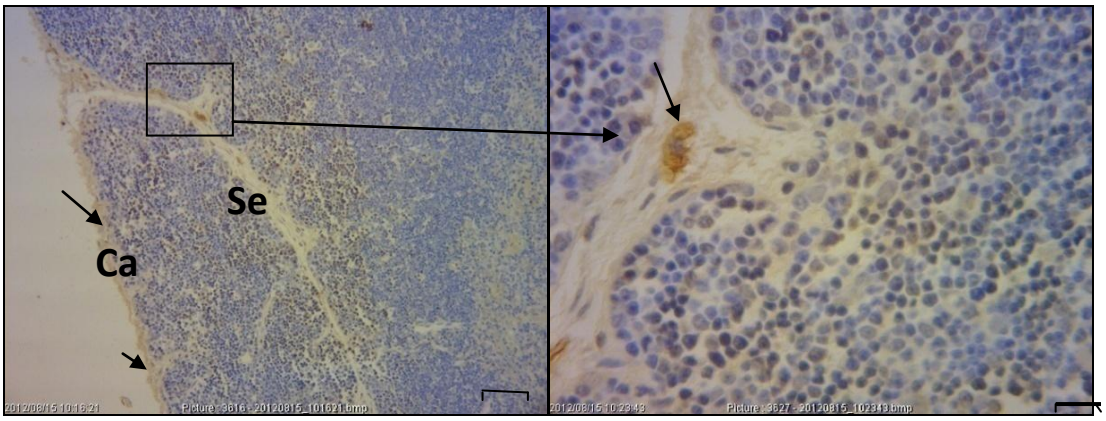


Plate 7 – Photomicrographs displaying the immunohistochemical staining of ChAT in paraffin embedded sections of thymus of Balb/C mice.
Fig 7a- Spleen is showing the localization of dark brown stained cells along the capsule and the interlobular septa indicated by arrows, scale bar= 45 µm, Fig 7b- The septa indicated in square of Fig 4.58a has been magnified and showing the IR of ChAT, Scale bars = 30µm, Ca-Capsule, Se-Septa

Table 1- Summarised immunoreactive values of VAChT and ChAT antigens

pecies	Immune tissue	Primary Abs / Subcellular compartments	VAChT	ChAT
Human	Spleen	Capsule	+	No reaction with human tissue
		Red pulp	++	
		White pulp	-	
	Lymph node	Capsule	+	
		Subcapsular, transverse sinuses	++	
		Paracortex	-	
		Medulla	-	
	Peyer's patches	Germinal centre	-	
		Mantle zone	-	
		Related tissue	-	
	Liver	Hepatocytes	-	
		Portal tract	-	
		Hepatic venules	-	
	Lymph agg. Of post.tong	Germinal centre	-	
		Mantle zone	-	

Wistar rats	Spleen	Capsule	+	+
		Red pulp	+	+
		White pulp	-	-
	Lymph node	Capsule	+	+
		Subcapsular, transverse sinuses	++	+
		Paracortex	-	-
		Medulla	-	-
	Peyer's patches	Germinal centre	-	-
		Mantle zone	-	-
		Related tissue	-	-
	Liver	Hepatocytes	-	-
		Portal tract	-	-
		Hepatic venules	-	-
	Thymus	Capsule	+	+
		Septa	++	+
		Cortex/medulla	+/-	+/-
Balb/C mice	Spleen	Capsule	+	+
		Red pulp	++	++
		White pulp	-	-
	Lymph node	Capsule	+	++
		Subcapsular, transverse sinuses	+	+
		Paracortex	-	-
		Medulla	-	-
	Peyer's patches	Germinal centre	-	-
		Mantle zone	-	-
		Related tissue	-	-
	Liver	Hepatocytes	-	-
		Portal tract	-	-
		Hepatic venules	-	-
	Thymus	Capsule	+	+
		Septa	++	+
		Cortex/medulla	-/+	+/-

IV. Discussion

The parasympathetic cholinergic input of immune tissues has been shown in our study by the application of ChAT & VAChT immunohistochemistry. The axon terminals of cholinergic neurons are concentrated with synaptic vesicles possessing VAChT proteins. Cholinergic nerve endings have been localized by revealing the IR of VAChT primary antibodies in those sites.

The VAChT immunoreactivity has been localized in the capsular surface, subcapsular areas and the septa as well as inside the cortical and corticomedullary junctions of the thymus used in our study. The findings produced regarding the IR of VAChT in this study is supported by findings shown in [9] in which they traced and localized the cholinergic IR fibres in the similar sites of rat thymus using AChE histochemistry. Reference [9] showed the AChE-positive nerve fibres which enter the capsular region of thymus and run parallel to blood vessels and in cortex and further those nerve fibres have been localized in the subcapsular and corticomedullary junctional areas. Along with the present results produced for the thymic distribution of VAChT proteins, ChAT immunohistochemistry study [4] also showed the fine perivascular nerve fibres localized in thymic parenchyma.

The VAChT proteins have been localized in the splenic tissue of murine and human species especially in the capsule and the red pulp. The trabeculae of venous sinuses found in the red pulp consist of tissue histiocytes as well as T lymphocytes which expressed higher IR of nicotinic and muscarinic AChRs in the cell surface and those results has been produced in one of our previous studies. The existence of AChRs in the above mentioned similar compartments further supports the localization of VAChT. While considering the nature of VAChT, their distribution was found to be similar in spleens of the three species concerned.

In accordance to the location of VAChT proteins in splenic compartments, AChE enzyme activity has been also identified in neuronal and non-neuronal compartments of spleen [11]. It was found primarily in lymphoid and reticular cells and to a lesser extent in nerve fibre like profiles in the splenic nerve, along central arterioles, in white pulp and in trabeculae [11]. Furthermore, findings shown in reference [6] who traced the autonomic innervation of spleen via the trans-synaptic retrograde tracer Pseudorabies virus (PRV) and shown the PRV positive neurons in the dorsal motor nucleus of the vagus, confirms the anatomical connections of spleen by the parasympathetic branches of the ANS.

In lymph nodes, the IR of VAChT has been localized in the subcapsular sinuses, trabeculae and medullary cords of the lymphoid tissues recovered from the three species in the present study. Further confirmation of the present findings need to be done by applying triple labelling of IF technique, in which the primary antibodies of selected AChRs and the VAChT will be used in the same tissue to visualise the co-expression of the IR.

The cholinergic innervation in lymph nodes has not been proved in past but the presence of Vasoactive Intestinal peptides (VIP) innervations in different sites of the tissue has been identified [11]. But the present study clearly shows the distribution of VAChT proteins in the capsular and subcapsular regions of lymph nodes. Therefore the present finding would encourage the researchers to seek further in identification of cholinergic nerves in lymph nodes.

Our results shows low intensity of VAChT immunoreactivity in the different compartments of the liver tissue recovered from Balb/C mice, Wistar rats and humans. In that case we could assume that the cholinergic nerves in hepatic parenchyma is poorly distributed or may be absent. Though functional significance of autonomic liver innervations was analysed [12], the cholinergic innervations of liver have not been well explained and the cholinergic intra hepatic nerve fibres have never been demonstrated morphologically up today [12, 13].

Studies regarding the autonomic innervations of fetal liver have not been carried out in the past. But there may be significant difference in the density of the nerve fibres in the adult and foetal liver depending on their age.

Similarly a faint IR of VAChT was observed in the germinal centres of Peyer's patches as well. The total IR in the specified regions of the three species was not significant and relatively very low or absent than the other immune tissues. Comparative studies were not available so far. Only peptidenergic fibres have been demonstrated in Peyers' patches [14, 15]. Recently a study [16] have showed the vagal nerve interactions in the intestinal wall in controlling the intestinal immune activation, but anatomical studies are needed to show the interactions.

The ChAT immunoreactive cells were localized in the lymphoid tissues of Balb/C mice and Wistar rats in this study. The IR of ChAT has been localized in the similar sites where the VAChT proteins were found. As the synthesis of ACh takes place predominantly in terminal boutons, ChAT enzyme localized in those relevant sites and ChAT immunohistochemistry proves to be a powerful tool in visualizing peripheral cholinergic structures [17].

In thymus, the IR of ChAT was observed in the capsule, septa and the cortex of the tissues of murine species and it was seen especially in the capsule and septal parts. Similar to our findings the results demonstrated in ChAT immunohistochemistry, showed the perivascular localizations in capsule, septa and in the parenchyma [4]. It has been further documented [5] and stated that in the subcapsular region of thymus, the parenchymal cholinergic fibres belong exclusively to phrenic nerve and branching seen in the deep cortical region, cortico-medullary junction and medulla. Also the catecholaminergic and cholinergic nerve fibres were detected along the vessels and parenchyma [5].

In murine splenic tissue, the IR of ChAT was clearly seen in the capsule and red pulp of the spleen and follows a similar pattern of distribution observed in localizing VAChT. The localization of the IR of VAChT, ChAT and evidence of distribution of AChRs superimpose in the same sites of the spleen confirms the cholinergic neural control of the immune responses. Further attempt should be taken to visualize the expression of the biomarkers in the same tissue using multiple labelling techniques of IHC.

In murine lymph nodes, the present study provides evidence of the ChAT IR in the capsule, subcapsular and medullary areas. The patterns of ChAT distribution in the immune tissues shows similarities of immunoreactive sites of VAChT, as well as the AChRs were found to be located at the same sites. When considering the liver and Peyer's patches, the ChAT immunoreactivity was relatively less and absent in some the subcellular compartments when compare to other cholinergic components.

The present anatomical findings of our study may help to understand the cholinergic neuro-immune interactions.

V. Conclusion

The close proximity of the distribution of VAChT and ChAT in capsular and perivascular supporting framework of these tissues confirms that the immune tissues receive cholinergic innervation and is through capsular and perivascular supporting framework of these tissues in Balb/C mice, Wistar rats and humans

VI. Acknowledgements

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